PATENT
Customer No. 22,852
Attorney Docket No. 06999.0005-01000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Catherine BLACKBURN et al.

Application No.: 09/359,672

Filed: July 23, 1999

For: DNA EXPRESSION IN TRANSFECTED CELLS AND ASSAYS CARRIED OUT IN TRANSFECTED CELLS

Group Art Unit: 1632

Examiner: Q. Nguyen

Commissioner for Patents
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Sir:

SUBMISSION OF PRIORITY DOCUMENT

In order to perfect the priority claim, Applicants submit a certified copy of British Patent application GB 9701492.2 filed January 24, 1997.

If any fees are necessary for the submission of these formal drawings, please charge our Deposit Account No. 06-0916.

Respectfully submitted,

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4. Title of the invention

Transfection Of Embryonic Stem Cells

5. Name of your agent *(if you have one)*

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Transfection Of Embryonic Stem Cells

This invention relates to transfection of embryonic stem cells (ES cells), to methods of transfecting ES cells so as to express a DNA, to a vector for transfection of ES cells and to transfected ES cells.

The wealth of sequence information now becoming available from the genome projects demands the development of new high throughput systems for functional analysis. A powerful route to discovering and characterising genes involved in determination and differentiation in mammals is potentially available via the genetic manipulation of embryonic stem (ES) cells *in vitro*.

ES cells, which are derived from the pluripotential inner cell mass (ICM) of the preimplantation mouse embryo(2,3), retain the capacity for multilineage differentiation both *in vitro*(4,5) and *in vivo*(6,7). In principle therefore gene products which influence developmental decisions should be assayable in ES cell culture systems, whatever the source of the cells. However, there are major difficulties in analysing cDNA function by ES cell transfection. The frequency of isolating stable transfectants is low ($<10^{-4}$ by electroporation, calcium phosphate co-precipitation or lipofection) and the great majority of transfectants show heterogeneous and unstable expression.

These problems are particularly significant in the case of cDNAs whose expression causes differentiation because differentiated ES cell progeny do not generally proliferate. In such cases transfectants may still be isolated but transgene expression will be minimal.

Episomal vectors have been used for functional screening in other cell types in order to increase the frequency of stable transfection and to achieve reliable transgene expression. However, previously described episomal vectors, for example based on Epstein-Barr virus (EBV) or bovine papilloma virus (BPV), have limitations both in host cell range and maintenance during long-term culture.

A modified extrachromosomal vector is known based on the replication system of murine polyoma virus(8). This plasmid, pMGD20neo, can be stably maintained as an episome in ES cells during long term culture. Importantly, the low levels of large T protein produced have no overt effect on the growth or differentiation properties of the ES cells (8,9). However, this vector already comprises two expression cassettes, one each for large T antigen and the neo selectable marker so its size constrains its use for expression of a third cassette containing a cDNA.

It is an object of the invention to provide a vector for transfection of an ES cell that results in expression that is more stable and more homogenous than hitherto attainable. A further object is to provide a method of expressing a DNA in an ES cell in a more stable and more homogenous manner than hitherto attainable. A further object is to provide for stable transfection of ES cells at a higher frequency than can be obtained using conventional vectors.

The invention is based upon the maintenance of a vector within an ES cell, wherein maintenance of the vector is dependant upon the continued presence within the cell of a certain factor and wherein that factor is not expressed by the vector but is produced in or present in the cell in an amount sufficient to maintain the vector.

Accordingly the invention provides, in a first aspect, a method of transfecting an ES cell, comprising:

(a) (i) transfecting the ES cell with a first vector that expresses a replication factor; or

(ii) otherwise obtaining an ES cell that expresses the replication factor;

and

(b) transfecting the ES cell with a second vector, wherein

(i) the second vector contains a DNA, or is adapted to receive a DNA, in operative combination with a promoter for expression of the DNA; and

(ii) extrachromosomal replication of the second vector is dependant

upon presence within the ES cell of the replication factor.

The replication factor is optionally non-toxic to the ES cell. Alternatively, the replication factor is toxic to the ES cell at high levels of expression but at low levels of expression is substantially non-toxic to the ES cell but at these low levels is present in sufficient amount to enable replication of the second vector.

Further, the replication factor preferably does not alter the ability of the ES cell to differentiate or proliferate, and may thus be regarded as being neutral to the ES cell phenotype. This enables the activities of the product of a cDNA to be investigated over a long time period and many cell generations without having to take account of possible interfering effects of the replication factor present within the ES cell. Again, the replication factor may be phenotype-neutral at all levels or may be neutral at a low level which is nevertheless a sufficient level to maintain the second vector within the ES cell.

The ES cell is preferably a mammalian cell, optionally a primate cell or a murine cell or an avian cell.

While reference is made to the second vector, it will be appreciated that the replication factor is optionally present in the cell other than following transfection with a first vector. For example a culture of ES cells that already express the replication factor may be obtainable from a third party.

In an embodiment of the invention described in detail below, the method comprises transfecting an ES cell with a first vector that expresses a viral replication factor, and thereafter transfecting the ES cell with a second vector that expresses a cDNA and is dependant upon presence of the viral replication factor for its extrachromosomal replication within the ES cell. The frequency of the first transfection step is generally low and may result in as few as 1 in 10^5 successful stable transfectants - this level of success is recognised as typical in this art. However, the second transfection has surprisingly and advantageously found to result in a significantly higher frequency of

successful stable transfectant colonies being obtained. The second transfection can be carried out with a 1% or higher success rate, which represents a 100-fold improvement over the art.

One suitable viral replication factor for mouse ES cells is polyoma large T antigen, in which case the ES cell of step (a) expresses the polyoma large T antigen and the second vector comprises an origin of replication that binds the polyoma large T antigen, such as the polyoma replication origin, referred to as *Ori*. Another suitable viral replication factor for primate cells is based upon Epstein Barr virus, in which the primate ES cell of step (a) expresses the EBNA-1 antigen and the second vector comprises an origin of replication that binds EBNA-1, such as *OriP*. Still further systems are optionally based on papilloma virus replication factors or SV40 virus large T antigen, and may also be selected from functional variants, derivatives and analogues of these replication factors, such as temperature sensitive variants.

In use, the second vector is constructed according to standard techniques so as to contain a cDNA sequence or insert of interest operatively combined with a promoter to express the cDNA. The second vector is used to transfect an ES cell already expressing a replication factor and successful transfectants are recovered in which it is found that the second vector is stably maintained within the ES cell and expresses the cDNA with a more homogenous pattern than when prior art techniques are followed. Thus, the invention provides an advantageous method for expression of a cDNA in an ES cell.

In this context, "homogenous" in relation to expression of a cDNA in a colony of transfected ES cells is used to indicate that most cells, or a large proportion of cells, or preferably most cells, or more preferably substantially all cells, express the cDNA and "stable" is used to indicate that the cells continue to express the cDNA at a similar level and preferably at substantially the same level. In the examples carried out to date and described below, homogenous transfection is seen with the method of the invention to a greater extent than in the art methods. Also, in the examples carried out to date and described below the method results in more stable expression,

meaning that expression does not alter over time. This has the advantage that study of the long term effects of a cDNA product is facilitated.

It is optional for the ES cell of step (a) first to be obtained or prepared by transfection of an ES cell by a first vector and for this then to be used for the starting ES cells for carrying out a plurality of separate transfections by second vectors containing different DNA inserts coding for different DNA products of interest. Following this procedure, the first transfection may be carried out with the level of success typically seen in conventional techniques and the ES cells obtained divided into separate colonies. The second transfection, introducing the DNA insert in the second vector, is then carried out with the higher levels of success typically seen in the methods of the invention.

In the case that the method comprises transfection with first and second vectors, it is preferable for the first vector to code for a selectable marker and for the second vector also to code for a selectable marker, though a different one. In a specific embodiment of the invention described below, the first vector codes for hygromycin resistance and the second codes for neomycin resistance. This allows selection of ES cells in which transfection by both first and second vectors has been successful.

It is a further embodiment of the invention for the method to comprise an additional transfection step with a third vector, wherein the third vector contains a cDNA, or is adapted to receive a cDNA, in operative combination with a promoter for expression of the cDNA, and extrachromosomal replication of the third vector is dependant upon presence within the ES cell of the replication factor. Transfection with the third vector is optionally at the same time as transfection with the second vector or subsequent thereto.

The second and third vectors preferably each comprise a selectable marker enabling selection of ES cells in which transfection has been successful. The respective selectable markers are preferably different if the method comprises transfection with both second and third vectors, and preferably different again from the selectable

marker of the first vector.

In a specific embodiment of the invention, the first vector is pMDG20neo and expresses polyoma large T antigen and the second vector comprises the natural target for polyoma large T antigen, namely *Ori*. In use, the large T antigen is expressed by the first vector and binds to *Ori* when the second vector enters an ES cell, thus enabling replication of the second vector and its maintenance within the ES cell in an extrachromosomal state. In successful transfectants, the vector remains extrachromosomal, and this is believed to render the vector relatively immune from effects seen when a vector is integrated into the host ES cell genome, which effect may include silencing of the cDNA resulting in unstable and heterogeneous expression.

An alternative to use of the first episomal vector is to introduce into the ES cell a construct that expresses the replication factor and integrates with the ES cell genome. The construct should therefore include a DNA sequence coding for the replication factor and means for selection of cells in which the construct has successfully integrated; one example is a construct that comprises cDNA coding for, in order, large T antigen - an internal ribosome entry site (IRES) - Bgeo. A culture of cells is then obtained by selecting for cells that express the selectable marker, such as in this case by selection in G418. Staining with Xgal is used to identify transfectant clones which show stable and homogenous expression. The construct preferably comprises a promoter that gives stable, low level expression in ES cells, such as the HMGCoA promoter. The cells obtained can then be subjected to transfection with the second and optionally the third vector.

In another embodiment of the invention the second vector comprises an inducible promoter. Some types of differentiated cells, derived from ES cells, can only be obtained with any reliability if a particular differentiating factor is expressed after a prior event. One example is neurone formation which generally only occurs after aggregation of cells. Thus, using an inducible promoter, expression of DNA that codes for the factor that leads to neurone formation can be controlled until the ES

cells have suitably aggregated. Interferon responsive promoters are some examples of inducible promoters. Alternatively, the cDNA is designed to be in a non-functional form and to be capable of being modified into a functional form at a later time. One possibility is for the cDNA to be disrupted for example by termination sequences which are flanked by target sites for a site specific recombinase, such as loxP sites, removable by Cre recombinase, or frt sites removable by Flp recombinase. Cre and Flp can be fused to steroid hormone receptors in order to make their activity regulatable. After administration of steroid the Cre or Flp recombinase will translocate to the nucleus and there convert the cDNA into a functional form by excision of the disrupting sequence.

The term DNA or cDNA is usually understood to refer to a DNA sequence that is transcribed into a mRNA that is translated into a polypeptide or protein. In the present invention the term is also intended to encompass any product of DNA expression. It thus includes DNA coding for an antisense RNA, or for an antisense ribozyme molecule.

The invention also relates to a vector. Accordingly, the invention provides in a second aspect, a vector for transfection of an ES cell, wherein:

(i) the vector contains a DNA, or is adapted to receive a DNA, in operative combination with a promoter for expression of the DNA;

(ii) extrachromosomal replication of the vector is dependant upon presence within the ES cell of a replication factor; and

(iii) the vector does not express the replication factor.

The vector is characterized in preferred embodiments as described above in relation to the second vector of the first aspect of the invention.

It is an advantage of the invention that very high efficiency of stable secondary transfection (supertransfection) of ES cells is obtained, for example transfection of ES cells harbouring pMGD20neo with a second plasmid containing the polyoma replication origin (*Ori*) (8).

The invention enables development of a series of vectors which give highly efficient and robust expression of transgenes in ES cells. Cloned cDNAs of interest can rapidly be characterised using this system. It should also be applicable to the discovery of novel regulatory molecules through functional expression screening of cDNA libraries.

Due to their pluripotent and proliferative character, key cellular processes such as viability, propagation, determination and differentiation, can be analyzed in transfected ES cells. The "supertransfection" system of the invention overcomes the limitations associated with conventional cDNA transfection and opens a powerful new route to gene discovery and characterisation in mammals.

Key features of the episomal supertransfection system, described according to the examples below, are that very high efficiencies of stable transfection are obtained and that cDNA expression is homogeneous, stable and reliably dictated by promoter strength. The increased efficiency of isolating stable transfectants is significant because it allows reliable detection of cDNAs whose expression results in cell death or differentiation. In addition a high transfection efficiency is generally advantageous for any high throughput assay system and is essential for functional cDNA library screening. The reliability of cDNA expression is critical for functional studies and the robust nature of expression from episomal vectors contrasts favourably with the variable and unstable expression observed in conventional ES cell transfectants.

Heterogeneous expression of integrated transgenes is not an artefact arising from use of bacterial *lacZ* as a reporter gene, firstly because similar observations have been made using mammalian thy-1 as a reporter in F9 cells, and secondly because ubiquitous expression of *lacZ* can readily be obtained following gene trap integrations (23,24). The expression pattern throughout the population cannot be determined by Northern blot but can only be revealed by *in situ* hybridization or use of a linked reporter gene such as IRES-*lacZ* (25) Heterogeneous expression, which previously occurred in the great majority of transfected clones following stable integration, gave unclear or misleading results on the phenotypic consequences of transgene

expression.

The difference in expression pattern between conventional transfectants and episomal supertransfectants of the invention arises because an extrachromosomal copy of a transgene is not subject to alteration during the integration process nor to modification arising from the genomic sequences flanking an integration site. The so-called "position effect" can modify both the level and pattern of transgene expression in stable transfectants. Furthermore, the expression of integrated transgenes is often suppressed over several generations in ES cell cultures. This silencing phenomenon contributes to the high backgrounds which can be obtained in double replacement type targeting strategies (26) . It has been observed in stable transfectants with different transgenes driven by viral promoters or minimal mammalian promoters such as the widely used human β-actin and mouse PGK-1 promoter elements. One hypothesis to explain this phenomenon is that transgenes may become targets of *de novo* methyltransferase in stem cells (27). Macleod et al. (28) reported that a methylation free locus could be generated in transgenic mice by introduction of the whole CpG island of the aprt promoter.

Whatever the molecular mechanism of silencing, it appears not occur to episomally maintained transgenes in vectors of the invention. In addition, the level of expression obtained from vectors of the invention is reliably dictated by promoter strength and can predictably be varied over at least a 10-fold range by appropriate choice of promoter. Episomal constructs of the invention thus offer considerable advantages for functional expression studies in ES cells.

Functional cDNA expression cloning is a powerful method for direct isolation of important genes. The expression screening approach has often been employed to isolate cDNAs encoding surface and secreted molecules via transient expression, for example in COS cells. In a few cases EBV-based systems have also been applied to isolate intracellular regulatory genes via stable expression in the target cells (29-32) . The high efficiency of supertransfection in the polyoma system of the invention indicates that this approach could be applied to functional cloning in ES cells. Based

On a transfection efficiency of 2.5%, a library of $5x10^5$ cDNA clones could be screened by electroporation of $2x10^7$ cells with 100μg DNA. For an effective library screen, the majority of transfectants should only take up a single plasmid. It is also advantageous if the cDNAs can readily be recovered in unrearranged form. Both of these conditions are satisfied by the episomal supertransfection system. By screening libraries prepared from undifferentiated ES cells it may be possible to isolate cDNAs whose products mediate self-renewal. In this case direct selection can be applied for colony formation in the absence of LIF. For cDNAs whose products direct differentiation, however, it will be necessary either to screen pools through several rounds or to incorporate an inducible promoter into the episome.

Recently, several improved protocols for *in vitro* differentiation of ES cells have been reported, which promote efficient generation of, for example, haematopoietic cells (33) , neurons (34) or cardiomyocytes (35) . The episomal expression strategy of the invention can be applied for gain-of-function assays and screens during these differentiation programmes. It can also be used for loss-of-function analyses via overexpression of anti-sense RNA or dominant-negative mutants. Combination of these differentiation systems with the episomal expression system will therefore provide powerful tools for analysing cell determination and differentiation events.

The invention is now described with reference to the accompanying drawings in which:

Figure 1 shows the structure of the episomal expression vector pHPCAG.

cDNAs can be introduced between two *Bst*XI sites using *Bst*XI adaptors. Abbreviations: ÆLT20: deleted polyoma large T expression cassette LT20; Pyori/enh: mouse polyoma virus replication origin and mouse polyoma mutant enhancer derived from F101 strain; SVpA: SV40 polyA addition signal; PGK*hph*pA: hygromycin B phosphotransferase gene expression cassette with mouse phosphoglycerokinase-1 (PGK) promoter and polyA addition signal; CAG: combined CAG expression unit; β-globinpA: rabbit β-globin polyA

addition signal; SVori: SV40 replication origin; ColE1ori: ColE1 replication origin; *amp*: *E.coli* β-lactamase gene conferring resistance to ampicillin.

Figure 2 shows supertransfection efficiency of pHPCAG in MG1.19 ES cells.

(A) Numbers of transfectant colonies per microgram of pHPCAG DNA.

$5x10^6$ MG1.19 ES cells were supertransfected with the indicated amounts of supercoiled pHPCAG followed by selection with hygromycin B for 8 days. The resulting number of drug-resistant colonies were scored and efficiency per μg DNA calculated.

(B) Total numbers of transfectant colonies plotted against total amount of plasmid DNA.

Figure 3 shows DNA hybridisation analysis of Hirt supernatants from supertransfectants.

Hirt supernatants were prepared from $5x10^6$ parental MG1.19 cells and pooled pHPCAG supertransfectants. 1/20 of each sample was digested with either *Eco* RI or *Hind*III and analyzed by filter hybridisation using a 344bp *Sca* I-*Ssp*I fragment from pUC19 which is common to both pMGD20*neo* and pHPCAG.

Figure 4 shows the effect of vector size on supertransfection efficiency.

20μg of each of the supercoiled vectors pLT20Æ*Nde*I*hph* (4.7), pLT20Æ*Bst*X1*hph* (5.5), pLT20Æ*Alw*NI*hph* (5.6), pLT20Æ*Sac*I*hph* (5.9), ptkp (6.2), pSV40e/p (6.4), PGK*hph*ÆLT20 (6.5), pmPGKp (6.6), phBAp (6.6), pHPCAG (7.7), ptkp-*lacZ* (8.9), pSV40e/p-*lacZ* (9.1), pmPGKp-*lacZ* (9.3), phBAp-*lacZ* (9.3), and pHPCAG-*lacZ* (10.4) were individually supertransfected into $5x10^6$ MG1.19 ES cells. The resulting numbers of hygromycin B resistant colonies were scored after 8 days. Transfection efficiencies are normalised

relative PGKhphÆLT20.

Figure 5 shows expression of β-galactosidase in MG1.19 transfectants.

Primary colonies were stained with Xgal after 8 days of selection.
(A) Typical homogeneous staining pattern obtained following supertransfection with supercoiled pHPCAG-*lacZ*.
(B) Heterogeneous staining pattern obtained in minority of clones following supertransfection with supercoiled pHPCAG-*lacZ*.
(C) Heterogeneous staining pattern typically observed following electroporation of linearized pHPCAG-*lacZ* and stable integration.
(D) Rare faint staining pattern obtained after supertransfection with supercoiled pHPCAG-*lacZ*.

Figure 6 shows the restriction pattern of plasmid DNAs recovered from pHPCAG-*lacZ* supertransfectant clone.

A supertransfectant MG1.19 clone carrying pHPCAG-*lacZ* was cultured for 60 days in the presence of hygromycin B. Hirt DNA was then prepared and electrotransformed into *E.coli* DH10B cells. Plasmid DNAs were recovered from transformants, digested with *EcoR*I, resolved by electrophoresis on 1.0% agarose gel and visualised by ethidium bromide staining. Expected fragment sizes: pMGD20neo, 4852bp and 2884bp; pHHPCAG-*lacZ*, 3697bp, 2810bp, 783bp and 397bp. Lane 1: size marker (1kb ladder:BRL); lane 2: control pMGD20; lane 3 : control pHPCAG-*lacZ*; lane 4: recovered pMGD20; lane 5,6: recovered pHPCAG-*lacZ*.

EXAMPLE 1

Materials and Methods

Vector constructions.

Standard recombinant DNA methods were used to construct all plasmids(10) .

Plasmid pHPCAG (Fig 1) was constructed from pMGD20neo(8) . The PGK*neo*polyA sequence was replaced by a hygromycin resistance marker, PGK*hph*pA, and large T sequences were deleted (see Results). A *Sal*I-*Sca*I fragment containing the CAG expression unit, a *Bst*XI stuffer sequence, the polyA addition signal derived from the rabbit β-globin gene and an SV40 replication origin (11) was inserted. Coding sequences for β-galactosidase, LIF or interleukin-2 were introduced between the BstXI sites.

For construction of episomal expression vectors with alternative promoters, the *Sal*I-*Xba*I fragment containing the CAG expression unit in pHPCAG-*lacZ* was replaced with the 344 bp SV40 enhancer/promoter (SV40e/p), the 466 bp human β-actin promoter (hBA), the 502 bp mouse phosphoglycerate kinase promoter (mPGK) and the 90 bp HSV-tk minimal promoter (tk), resulting in pHPSV40e/p-*lacZ*, pHPhBA-*lacZ*, pHPmPGK-*lacZ* and pHPtk-*lacZ*, respectively.

Episomal vectors with alternative selection markers were constructed by replacing the PGK*hph*pA cassette in pHPCAG with the SV*bsr*pA cassette carrying the *E.coli* blasticidin S deaminase (*bsr*) gene derived from pSV2*bsr* (Waken Seiyaku) or the hCMVzeopA cassette carrying the *Streptoalloteichus* bleomycin resistant gene (*Sh ble*) derived from pZeoSV (Invitrogen) to generate pBPCAGGS and pZPCAGGS, respectively.

Cell culture and transfection.

MG1.19 ES cells are derivatives of the CCE line which stably maintain around 20 episomal copies of pMGDneo(8) . They were maintained on gelatin-coated plates in Glasgow modified Eagle's medium (GMEM, Gibco-BRL) supplemented with 10% fetal calf serum, 0.1 mM β-mercaptoethanol, non-essential amino acids, 200 μg/ml G418, and 100U/ml LIF produced in COS-7 cells(11,12) . For supertransfection, routinely, $5x10^6$ MG1.19 cells were suspended in 800 μl of PBS, incubated with 20 μg of supercoiled vector DNA for 10 min on ice, and electroporated at 200V/960μF using a Bio-Rad gene pulser. Cells were transferred into gelatinized plates and allowed to recover overnight before addition of appropriate selection agent. Histochemical

staining for β-galactosidase was carried out with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (13) , and β-galactosidase activity was measured by incubation of cell extracts with o-nitrophenyl-β-D-galactopyranoside (ONPG). Differentiation was induced in monolayer culture as described (12) .

Analysis of episomal vectors in the supertransfectants.

Hirt supernatants were prepared as described (14) . For amplification of recovered episomal vectors, electrocompetent *E. coli* DH10B cells were transformed by electroporation at 2500V/25μF/200½.

Results

Construction of an episomal expression vector.

Polyoma-based plasmids have recently been reported to be competent for episomal propagation in ES cells (8) . The plasmid pMGD20neo contains a modified large T expression unit called LT20, the viral origin of replication (*Ori*), and the PGKneopA cassette as a selectable marker. This plasmid can be maintained as an extrachromosomal element in wild-type ES cells. It can be modified to include a cDNA expression unit (9) . However, the low frequency of conventional stable transfection of ES cells (Å 1 x 10^{-5}) remains a limiting feature. Furthermore, episomal propagation only occurs in 10-15% of primary transfectants (8,9) .

A second plasmid has been described which can be maintained as an episome only in ES cells which independently express the large T protein (8) . This plasmid, PGKhphÆLT20, contains LT20 with a large deletion in its coding sequence, *Ori*, and PGK*hph*pA as a selectable marker. When introduced into a cell line such as MG1.19, in which episomal maintenance of pMGDneo has already been established, the yield of hygromycin B resistant stable transfectants is extremely high. This phenomenon of supertransfection is presumed to arise from the pre-existence of large T protein in the recipient cells.

In the studies reported below the modification and use of supertransfection vectors for cDNA expression is characterised.

Size of vector

PGK*hph*ÆLT20 retains part of the large T coding sequence. We made a series of deletions in the ÆLT20 sequence to minimize the vector size and thereby increase the capacity for inserts and reduce potential bias in the construction and screening of cDNA libraries. The supertransfection efficiency of four derivative plasmids was then compared in MG1.19 cells. All showed comparable supertransfection efficiency to PGK*hph*ÆLT20 (data not shown). The smallest, pLT20Æ*Ndehph*, has a deletion of 2953 bp, yielding an episomal vector backbone of only 4.7kb.

Expression unit

Into this minimal episomal vector we introduced a cDNA expression unit. Transcriptional initiation signals are supplied by the CAG cassette(11) , which comprises the human cytomegalovirus immediate early enhancer, a 1kb fragment of the chicken β-actin gene (promoter, non-coding first exon and first intron), and a splice acceptor derived from the rabbit β-globin gene. This combination has been shown to direct strong expression of cDNAs in undifferentiated stem cells. The resulting expression vector, pHPCAG (Fig 1), contains the CAG sequences followed by the *Bst*XI stuffer sequence derived from pCDM8 as a cDNA cloning site, and a polyA addition signal derived from the rabbit β-globin gene. In addition the plasmid contains the PGK*hph*pA(15) cassette for hygromycin selection of ES cell transfectants, the polyoma*Ori* with pyF101-derived mutant enhancer element(16) for stable episomal replication in cells expressing polyoma large T protein, and the β-lactamase (*amp*) gene and prokaryotic replication origin for amplification in *E. coli*. The SV40 *Ori* is also present to allow for transient episomal replication in mammalian host cells expressing SV40 largeT, such as COS cells(17) .

Characterization of supertransfection.

The parameters of supertransfection with pHPCAG and derivatives were investigated. First, $5x10^6$ MG1.19 cells were electroporated with various amount of supercoiled pHPCAG, selected in medium containing 80 μg/ml of hygromycin B for 8 days, and the number of stem cell colonies scored after Leishman's staining(12) . Although the highest efficiency per μg DNA was observed with minimum amounts (1-2 μg) of

ector DNA (Fig. 2B), the total yield of hygromycin B resistant colonies increased with increasing amount of plasmid (Fig 2A). Saturation was not reached over the range of plasmid concentrations tested. With 100 μg plasmid DNA, 150,000 hygromycin B-resistant colonies were obtained, representing 3% of total treated cells. Disablement for episomal replication by linearisation of pHPCAG prior to electroporation reduced this transfection efficiency to less than 0.01%.

Next, increasing numbers of MG 1.19 cells were subjected to electroporation with 100 μg of pHPCAG DNA. Comparable stable transfection efficiencies in the range 3-6% were obtained with up to $2.5x10^7$ cells.

The copy number of pHPCAG in the supertransfectants was analyzed by preparation of Hirt supernatants followed by filter hybridisation. This analysis revealed that supertransfected cells carried approximately 20 copies each of pMGDneo and pHPCAG (Fig. 3).

These data demonstrate that the efficiency of supertransfection with pHPCAG is extremely high. However, episomal vectors can be limited in their capacity for inserts because increased size may cause inefficient replication or instability. To investigate this issue in the ES cell system, episomal vectors of different size were supertransfected into MG 1.19 cells. The numbers of supertransfectant colonies were scored and plotted against vector size (Fig. 4). These data indicate that there is a progressive reduction in transfection efficiency with increasing plasmid size. In particular, the largest plasmid tested, a derivative of pHPCAG with a 3kb *lacZ* insert (total size 10.4kb) showed a 50% reduction in colony number. However, that this may not be due entirely to the size of the plasmid because the very high levels of β-galactosidase expression may exert some toxic effects (see below).

***lacZ* expression in supertransfectants.**

To evaluate the level and pattern of expression of transgenes from pHPCAG, the *E.coli* β-galactosidase (*lacZ*) gene was introduced into this vector. The resulting vector, pHPCAG-*lacZ*, was introduced into MG1.19 cells and supertransfectants

isolated by selection with 80 μg/ml of hygromycin B for 8 days. The number of colonies isolated was 50% of the number obtained in a parallel supertransfection with pHPCAG (see above). The colonies were smaller and many of the cells showed an abnormal spindle-shaped morphology. These effects were not observed with several other inserts in pHPCAG and are suggestive of a toxic effect of the high level *lacZ* expression. The primary supertransfectants were stained with X-gal and the staining pattern examined under phase-contrast microscopy. Staining was detectable after 5 minutes incubation and was intense by 1 hour. This level of β-galactosidase activity is significantly higher than we have observed from a variety of integrated expression constructs.

Approximately 80% of supertransfectant colonies showed ubiquitous expression (>90% cell positive) as shown in Fig.5-A (i). Of the remainder, 15% showed heterogeneous expression (Fig.5-A (ii)), and 5% showed little or no staining (Fig.5-A (iv)). The latter two classes are likely to arise as a result of vector integration which occurs in up to 20% of supertransfectants (8). In transfectants derived by electroporation of linearized pHPCAG-*lacZ* into MG1.19 cells (which results in vector integration in the majority of clones), only 15 % of colonies showed homogeneous staining whereas 70% of colonies stained heterogeneously (Fig.5-A (iii)), and 15% showed no expression.

Analysis of expanded clones from each class of transfectant established that this difference in expression characteristics was stable. Twelve of 13 expanded supertransfectants expressed *lacZ* homogeneously. In contrast, only 4 out of 24 clones derived using linearized vector showed homogeneous expression. This is consistent with our previous observations on integrated expression constructs in ES cells. In fact the CAG unit gives a significantly higher frequency of colonies which show stable ubiquitous expression than other promoters we have examined.

The difference in staining pattern between episomally maintained and integrated vectors indicates that the former escape modifying influences arising from integration and reliably give full activity of the expression unit.

Comparison of expression with various promoters on episomal vector.

An ability reliably to generate predetermined levels of expression would be a important attribute for a transgene expression system. The previous observations suggested that episomal vectors offered potential to achieve unmodified expression. Various promoters with different strengths in undifferentiated stem cells were therefore introduced into the episomal vector by replacing the CAG expression unit of pHPCAG-*lacZ*. Expression of the *lacZ* reporter was then assayed in both transient and stable supertransfectants (Table 1). The relative ratio of β-galactosidase activity obtained from the SV40 enhancer/promoter complex, the human β-actin promoter, the mouse PGK-1 promoter and the HSV-tk minimal promoter in transient transfectant was retained in stable supertransfectants. The CAG expression unit showed strongest activity in the tested constructs in both transient and stable transfectants. In this case, however, the relative ratio in transient transfectants, 19 times higher than SV40, was significantly reduced in stable transfectants. This may arise from an elimination of strong expressants due to a toxic effect of high *lacZ* expression (see above). A reduced number of supertransfectants and smaller size of colonies was observed only with the CAG vector.

Stability of supertransfected episomal expression vector during long-term culture and differentiation of host cells.

A critical limitation of previously described episomal vectors is their instability during long-term culture. Many episomal vectors undergo integration into the host genome after long-term culture, resulting in a reduction in expression and inability to recover transgenes by preparing Hirt supernatants. To test the stability of the supertransfection system, four pHPCAG-*lacZ* supertransfectant clones were cultured for 60 days (approximately 90 generations) under continuous selection with 80 μg/ml of hygromycin B. Three of the four clones maintained relatively constant levels of β-galactosidase activity determined by ONPG assay and uniform expression as revealed by Xgal staining. The fourth clone showed unstable and variegated expression, as commonly observed on vector integration. Hirt supernatants were prepared from one of the stably expressing clones at the end of the 60 day culture period. Filter hybridization analysis of the Hirt DNA indicated that the ES cells carried

Approximately 20 copies of pMGD20 and 5 copies of pHPCAG-*lacZ* per cell (data not shown). The lower copy number of pHPCAG-*lacZ* may be due to its larger size and/or the toxic effect of strong *lacZ* expression. The Hirt DNA was transformed into *E.coli* for further analysis. Of the bacterial transformants, 20% carried pHPCAG-*lacZ* and the remainder carried pMGD*neo*20, in good agreement with the hybridization data. Restriction mapping showed no evidence of rearrangement in either plasmid (Figure 6).

In the experiment above, cells were maintained under selection with hygromycin B. In the absence of selection pressure, supertransfectant clones lost expression of β-galactosidase over several passages in culture. This might indicate an intrinsic instability of supertransfected episomal vectors. However, it could also reflect a selective disadvantage for ES cells which express high levels of β-galactosidase. It is noteworthy in this regard that the primary episome, pMGD20neo, is stable in the absence of selection(8) .

Stability of expression from pHPCAG-*lacZ* during the *in vitro* differentiation of ES cells was also analyzed. Differentiation was induced in three ways: withdrawal of LIF; exposure to retinoic acid; and treatment with 3-methoxybenzamide(18) . After 6 days the differentiated progeny stained ubiquitously in all three cases (data not shown).

These data indicate that supertransfected episomal vectors can be maintained in an extrachromosomal state and direct strong expression of transgenes during long-term self-renewal and differentiation *in vitro*.

Production and secretion of the cytokine LIF from an episomal ES cell expression vector.

The pHPCAG-*lacZ* plasmid can efficiently direct strong and homogeneous expression of the cytoplasmic *lacZ* reporter gene. We next investigated expression of a secreted molecule, the cytokine LIF. LIF is an essential supplement to ES cell culture medium because it inhibits differentiation of the stem cells (19,20) . Expression of LIF can readily be assayed by formation of stem cell colonies in media lacking the cytokine.

Episomal vectors for expression of another cytokine, interleukin-2 (which has no effect on ES cell phenotype), and for LIF were electroporated in parallel into MG1.19 cells. The cells were seeded at low density ($1.5x10^4$ and $5x10^3$ cells per 90mm plate) to avoid the rescue effect which arises from the production of LIF by differentiated ES cell progeny (21) , and cultured with 80 μg/ml of hygromycin B for 8 days. pHPCAG-*il2* generated large numbers of stem cell colonies in medium supplemented with LIF, but none in the absence of LIF. pHPCAG-*lif* in contrast produced comparable numbers of healthy stem cell colonies in both the presence and absence of exogenous LIF (Table 2). These colonies could be expanded and propagated without LIF-supplementation of the medium. These data confirm previous observations that increased autocrine expression of LIF renders ES cells factor-independent (22) and establish that secreted proteins are produced efficiently and stably by this episomal expression system.

Co-supertransfection of episomal vectors.

Introduction of two or more different transgenes into cells is often required for analysis of protein interactions and/or co-operative function. The poor efficiency of homogeneous expression in conventional transfectants is a major obstacle for such investigations in ES cells. To test the possibility that the episomal approach could be applied to co-express multiple cDNAs, we constructed episomal expression vectors with different selection markers. Co-supertransfection of episomal vectors was then assessed.

The basic episomal expression vector pHPCAG carries the hygromycin phosphotransferase gene driven by mouse PGK-1 promoter (PGK*hph*pA). We prepared episomal vectors which carry the zeocin-resistance gene driven by the human cytomegalovirus immediate-early promoter (pZPCAG), or the blasticidin S-resistance gene driven by the SV40 enhancer/promoter (pBPCAG) by substitution of the PGK*hph*pA cassette in pHPCAG. These vectors were supertransfected into MG1.19 cells followed by 8 days selection with the appropriate antibiotic. Comparison of the numbers of resulting drug-resistant colonies (Table 3) revealed

that these selection systems are slightly less efficient than hygromycin B selection but nonetheless enable large numbers of supertransfectants to be isolated.

ES cells harbouring two different episomal vectors can be isolated by repeated supertransfection. Supertransfectants carrying pHPCAG can be transfected again with pBPCAG or pZPCAG, with comparable efficiency to the original supertransfection into MG1.19 ES cells (data not shown). This should allow establishment of efficient screens for assaying functional interactions between gene products.

The effects of co-electoporation of supertransfection vectors were also investigated. pHPCAG (10 μg) and pBPCAG (10 μg) were co-electroporated into $5x10^6$ MG1.19 cells. Cells were selected in hygromycin B or blasticidin S only, or both, for 8 days and the number of drug-resistant colonies scored in each case. The numbers of hygromycin or blasticidin S single-resistant colonies were 39,000 and 13,000, respectively, while the number of double-resistant colonies was 1,200. Thus the apparent efficiency of incorporation of both plasmids was less than 10%. Similar results were obtained on co-supertransfection of pHPCAG and pZPCAG (not shown). These data suggest that the majority of supertransfectants incorporate only one plasmid under these electroporation conditions. This is significant for application of the episomal system to functional cDNA library screening.

EXAMPLE 2

The effects of overexpression of a large number of transgenes in ES cells were investigated by construction of vectors based on pHPCAG and including a DNA insert coding for the transgene being investigated. 5×10^6 ES MG1.19 cells were supertransfected with 20 μg of expression vectors and selected with 80 μg/ml of hygromycin B for 8 days. The numbers of drug-resistant colonies were counted and normalised relative to numbers obtained with empty vector. The results are shown in Table 4.

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We have thus described the development of an optimised transfection and expression system which will enable high throughput functional screening of cDNAs in pluripotential mouse embryonic stem (ES) cells and differentiated derivatives. The strategy is based on extrachromosomal vector replication driven by expression of polyoma large T protein. When a vector containing a polyoma origin of replication is introduced into an ES cell line that harbours polyoma large T antigen, a high frequency of stable secondary transfection results. This process is referred to as supertransfection. Supertransfected plasmids can be maintained episomally during long-term culture and during differentiation *in vitro*. Expression of a β-galactosidase reporter from an episomal vector is both ubiquitous and stable, in contrast to the variegated and unstable expression usually observed after cDNA integration into the ES cell genome. Moreover, in the absence of integration, promoter strength is predictable and a range of expression levels can reliably be achieved by using different elements. We also show that episomal vectors can be used for efficient expression of both cytosolic and secreted proteins. These features should make this system invaluable for functional analyses of defined cDNAs and for direct expression screening of cDNA pools or libraries in ES cells.

Table 1. Comparison of β-galactosidase activities directed by various promoters in transient and stable supertransfectants.

Promoter	Relative β-gal activity	
	transient	stable
SV40 e/p	1.0	1.0
hβAp	1.1	0.7
mPGKp	0.5	0.5
TKp	0.1	0.1
CAG	19.0	1.8

$5x10^6$ MG1.19 ES cells were supertransfected with 20μg of vector DNAs. After 3 days culture for transient expression assay or 8 days selection with hygromycin B for stable expression assay, the β-galactosidase activity generated by these constructs was measured by ONPG assay. Results are normalised relative to activity generated by the SV40e/p construct. See 'Materials and methods' for construction details of vectors.

Table 2. Supertransfection of LIF and IL-2 expression vectors into MG1.19 ES cells.

Vector	LIF in medium	No. of hyg^r stem cell colonies
pHPCAG-*lif*	+	42,000
pHPCAG-*lif*	-	38,000
pHPCAG-*il2*	+	48,000
pHPCAG-*il2*	-	0

$5x10^6$ MG1.19 ES cells were supertransfected with 20μg of vector DNAs. After 8 days selection with 80μg/ml of hygromycin B in the presence or absence of LIF, the number of stem cell colonies were scored.

Table 3. Efficiency of supertransfection of vectors with various selection markers.

Selection marker	Drug for selection (μg/ml)	No. of resistant colonies
PGK*hph*pA	hygromycin B (80)	50,000
SV*bsr*pA	blasticidin S (4)	12,600
hCMV*zeo*pA	zeocin (20)	20,600

$5x10^6$ MG1.19 ES cells were supertransfected with 20μg of vector DNAs of episomal vectors, pBPCAG and pZPCAG, which carry *bsr* and *zeo* resistance genes respectively. After 8 days selection with the appropriate drug, the number of drug-resistant stem cell colonies were scored.

Table 4. Effects of overexpression of transgenes in ES cells using pHPCAG.

cDNA	Relative number of hygro^R colonies	Colony Size and Morphology
None	1.00	Normal
lacZ	0.64	small
DIA/LIF	0.87	slightly small
IL-2	0.92	slightly small
Rex-1	0.88	Normal
Fgf-2	0.65	Normal
Fgf-4	0.82	Normal
Fgf-5	0.41	Normal
Oct-1	0.17	small
Oct-2	0.65	slightly small
Oct-3/4	0.61	differentiated
Oct-6	0.03	some differentiation
c-jun	0.47	small
E1A	0.08	differentiated
Jak2 K/E	0.75	Normal
bcl-2	0.28	small, spindle morphology
MAPKP	1.38	Normal
RXRα	0.20	some differentiation
RXRβ	0.63	Normal
RXRγ	0.91	Normal
COUP-TF1	0.40	some differentiation
HNF-4	0.05	Normal
Stat1	0.10	small
Stat3	0.52	Normal
Stat4	0.16	Normal
Stat3DON*	0.14	differentiated

$5x10^6$ ES MG1.19 cells were supertransfected with 20 μg of expression vectors and selected with 80 μg/ml of hygromycin B for 8 days. The numbers of drug-resistant colonies were counted and normalised relative to numbers obtained with empty vector.

*Stat3DON is the dominant interfering mutant form of Stat3 described by Akira *et al.* (1996).

Claims

1. A method of transfecting an ES cell, comprising:

   (a) (i) transfecting the ES cell with a first vector that expresses a replication factor; or

   (ii) otherwise obtaining an ES cell that expresses the replication factor;

   and

   (b) transfecting the ES cell with a second vector, wherein

   (i) the second vector contains a DNA, or is adapted to receive a DNA, in operative combination with a promoter for expression of the DNA; and

   (ii) extrachromosomal replication of the second vector is dependant upon presence within the ES cell of the replication factor.

2. A method according to Claim 1 wherein the replication factor is a viral replication factor.

3. A method according to claim 1 or 2 wherein the viral replication factor is selected from polyoma large T antigen, EBNA-1 antigen, papilloma virus replication factors, SV40 large T antigen and functional variants, analogues and derivatives thereof.

4. A method according to any of claims 1-3 wherein the second vector does not express the replication factor.

5. A method according to any of claims 1-4 wherein the second vector expresses a selectable marker.

6. A method according to any of claims 1-5 further comprising transfecting the ES cell with a third vector, wherein the third vector contains a DNA, or is adapted to receive a DNA, in operative combination with a promoter for expression of the DNA, and replication of the third vector is dependant upon presence within

the ES cell of the replication factor.

7. A method according to claim 6 wherein transfection with the third vector is carried out subsequently to transfection with the second vector.

8. A method according to claim 6 wherein transfection with the third vector is carried out simultaneously with transfection with the second vector.

8. A method according to any of claims 6-8 wherein the third vector expresses a selectable marker, which selectable marker is different to that expressed by the second vector.

9. A method according to any preceding claims wherein the ES cell of step (a) expresses polyoma large T antigen and the second vector comprises a natural target for polyoma large T antigen, such as *Ori* or functional variants thereof adapted to bind to polyoma large T antigen.

10. A method according to any preceding claims wherein the first vector expresses polyoma large T antigen.

11. A method according to any preceding claim wherein the DNA codes for a polypeptide or protein.

12. A method according to any of claims 1-11 wherein the DNA codes for an antisense DNA.

13. A method according to any preceding claims wherein the promoter is inducible.

14. A vector for transfection of an ES cell, wherein:

(i) the vector contains a DNA, or is adapted to receive a DNA, in operative combination with a promoter for expression of the DNA;

(ii) extrachromosomal replication of the vector is dependant upon presence

within the ES cell of a replication factor; and

(iii) the vector does not express the replication factor.

15. A vector according to Claim 14 wherein the replication factor is a viral replication factor.

16. A vector according to claim 14 or 15 wherein the viral replication factor is selected from polyoma large T antigen, EBNA-1 antigen, papilloma virus replication factors, SV40 large T antigen and functional variants, analogues and derivatives thereof.

17. A vector according to any of claims 14-16 wherein the vector expresses a selectable marker.

18. A vector according to any of claims 14-17 comprising a natural target for polyoma large T antigen, such as *Ori* or functional variants thereof adapted to bind to polyoma large T antigen.

19. A vector according to any of claims 14-18 wherein the DNA codes for a polypeptide or protein.

20. A vector according to any of claims 14-18 wherein the DNA codes for an antisense DNA.

21. A vector according to any of claims 14-20 wherein the promoter is inducible.

22. Use of a vector according to any of claims 14-21 for expression of a DNA sequence within an ES cell.

23. An assay for the effect of expression in an ES cell of a protein or polypeptide, comprising the steps:

(a) (i) transfecting the ES cell with a first vector that expresses a

replication factor; or

(ii) otherwise obtaining an ES cell that expresses the replication factor;

(b) transfecting the ES cell with a second vector, wherein

(i) the second vector contains a DNA coding for the protein or polypeptide in operative combination with a promoter for expression of the DNA;

(ii) the second vector also contains a DNA coding for a selectable marker in operative combination with a promoter for expression of the selectable marker; and

(iii) extrachromosomal replication of the second vector is dependant upon presence within the ES cell of the replication factor;

(d) selecting for ES cells that have been transfected with the second vector; and

(c) maintaining the selected ES cells over a plurality of generations so as to assay the effect of expression of the protein or polypeptide.

24. An assay according to claim 23 wherein step (a) is carried out once and the ES cells obtained are divided and used for a plurality of separate assays in which steps (b)-(d) are carried out a plurality of times with second vectors containing different DNA sequences.

Figure 1

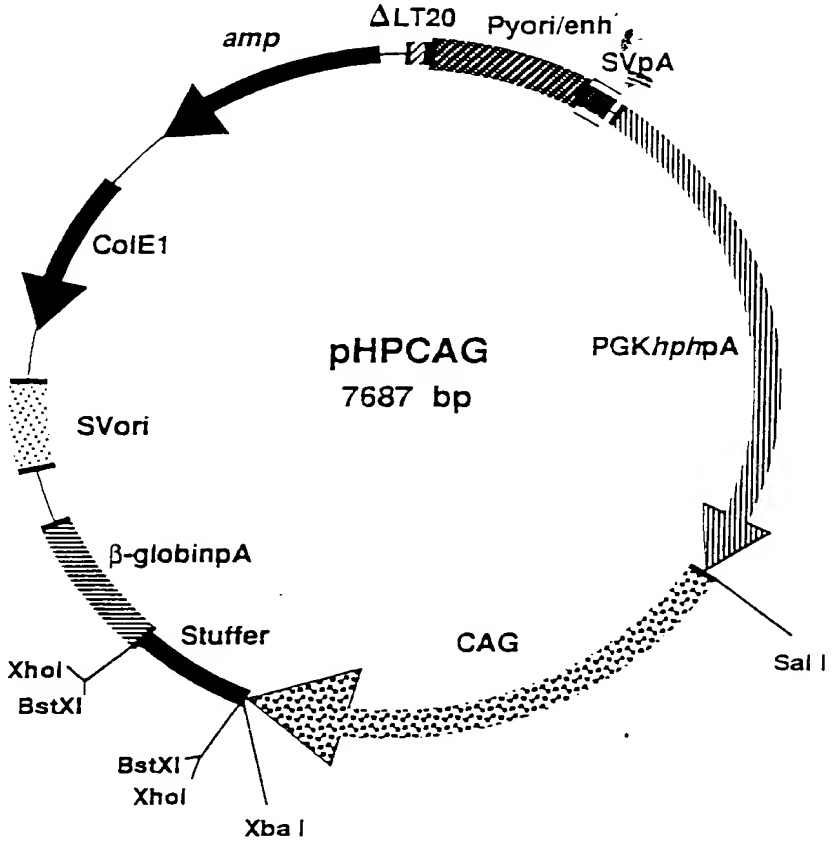
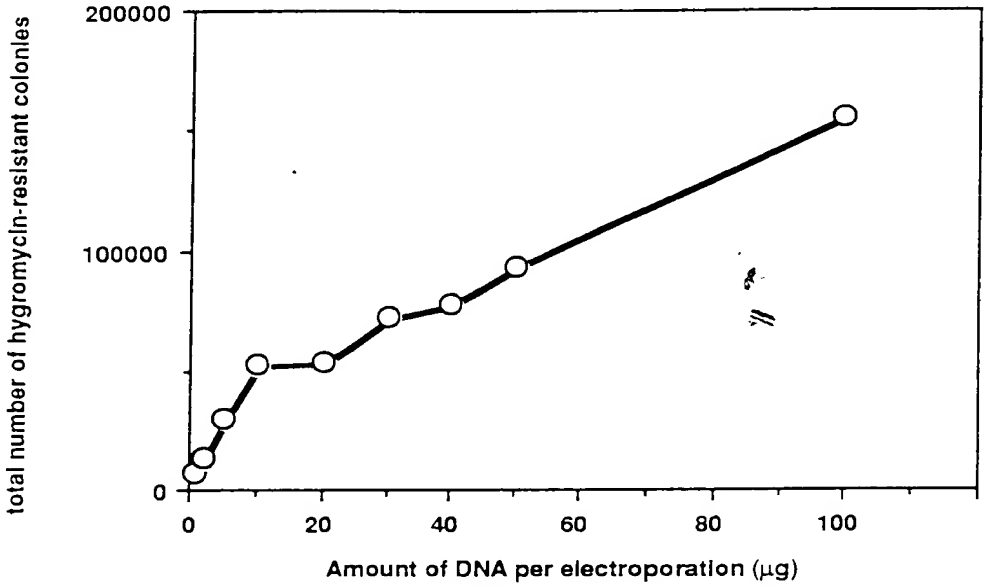

ΔLT20
Pyori/enh
SVpA
amp
ColE1
pHPCAG
7687 bp
PGKhphpA
SVori
β-globinpA
Stuffer
CAG
Sal I
XhoI
BstXI
BstXI
XhoI
Xba I

Figure 2

A


total number of hygromycin-resistant colonies
200000
100000
0
0
20
40
60
80
100
Amount of DNA per electroporation (µg)

B

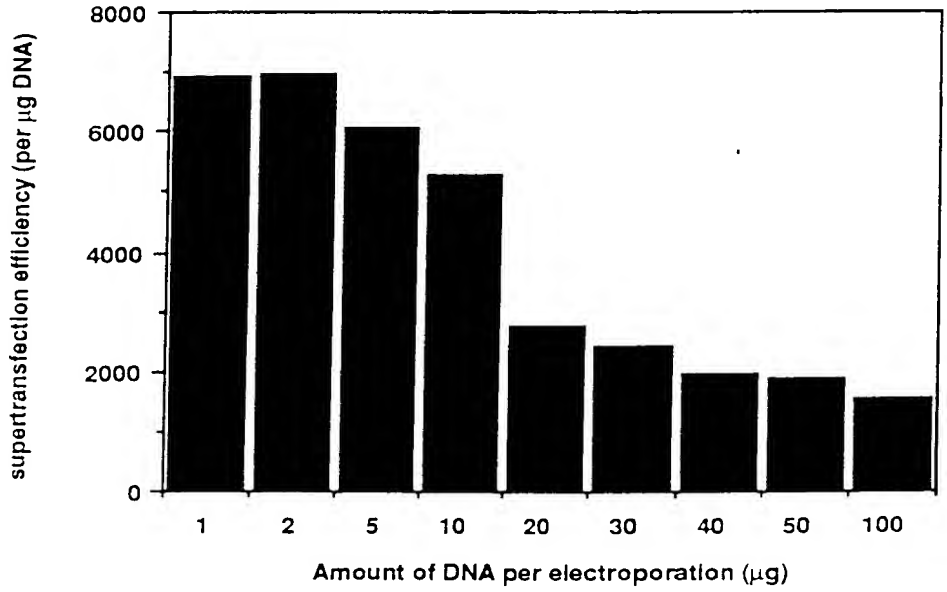

supertransfection efficiency (per µg DNA)
8000
6000
4000
2000
0
1
2
5
10
20
30
40
50
100
Amount of DNA per electroporation (µg)

Figure 3

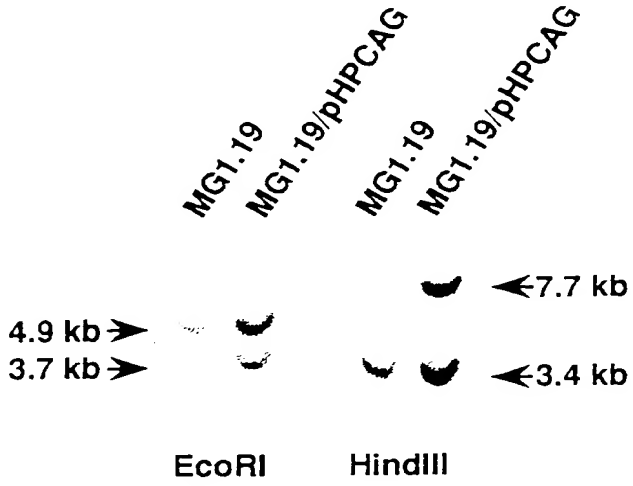

MG1.19
MG1.19/pHPCAG
MG1.19
MG1.19/pHPCAG
7.7 kb
4.9 kb
3.7 kb
3.4 kb
EcoRI
HindIII

Figure 4

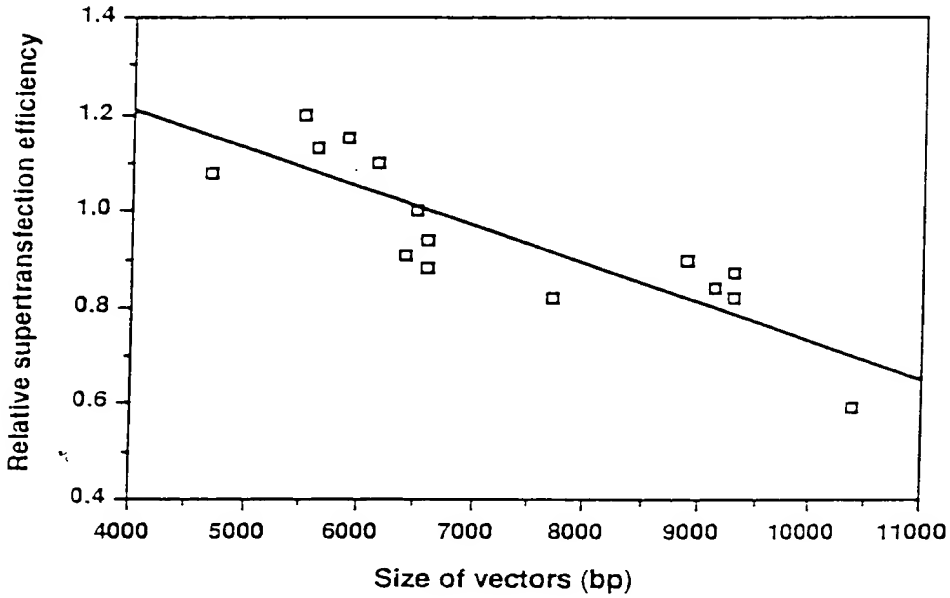

Relative supertransfection efficiency
1.4
1.2
1.0
0.8
0.6
0.4
4000
5000
6000
7000
8000
9000
10000
11000
Size of vectors (bp)

Figure 5

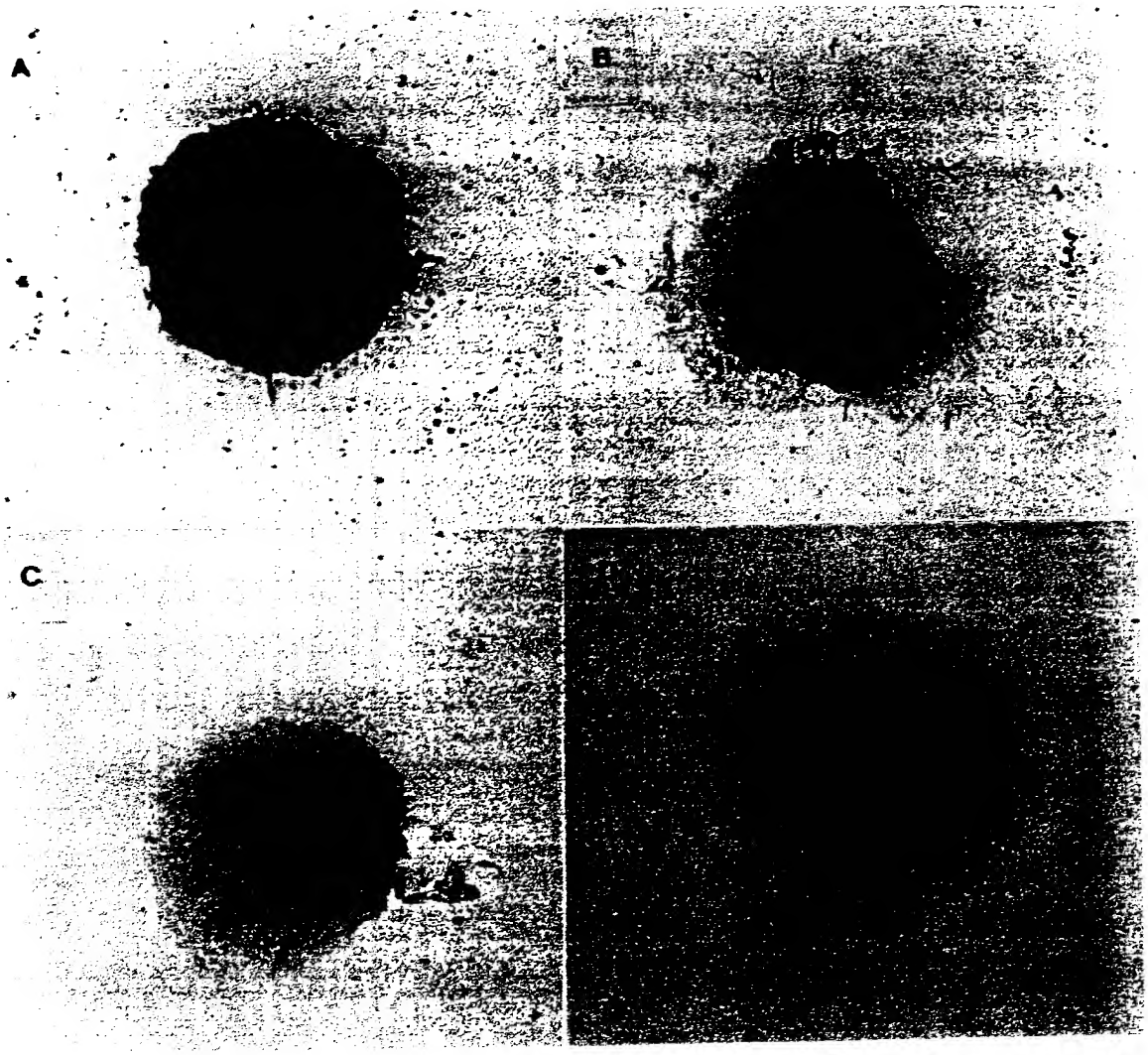


Figure 6